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Strong Inverse Correlation Between Serum TPO Level and Platelet Count in Essential Thrombocythemia

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Serum thrombopoietin (TPO) levels in 50 essential thrombocythemia (ET) patients were measured using a highly sensitive sandwich ELISA. In nine cases, TPO levels were measured at two points with different platelet counts. ET patients showed significantly higher serum TPO levels (n = 59, 2.70 ± 2.74 fmol/mL, $P < 0.0001$) than those of normal individuals (n = 29, 0.83 ± 0.36 fmol/mL). Twenty-three previously untreated ET patients also showed significantly higher serum TPO levels (1.33 ± 0.75 fmol/mL, $P = 0.0066$) than normal individuals. Extremely high serum TPO levels (5.46 ± 3.68 fmol/mL) were observed in ET patients with normal platelet counts. Furthermore, a strong inverse correlation was found between serum TPO levels and platelet counts in ET patients ($R = -0.729$, $P < 0.0001$). This inverse correlation also held for each of nine cases with two-point TPO measurements. In the clinical course of ET, megakaryocyte mass may parallel the platelet mass before and after chemotherapy. Although it is unknown whether overproduction of TPO exists or not in ET, total platelet and megakaryocyte mass, i.e., the total number of c-Mpl, may play a role to regulate serum TPO levels. Am. J. Hematol. 63:131–135, 2000.

Key words: essential thrombocythemia; thrombopoietin; platelet

INTRODUCTION

Essential thrombocythemia (ET) is a clonal myeloproliferative disorder (MPD) manifested by the deregulated proliferation of pluripotent stem cells resulting in selective expansion of megakaryocyte progenitor cells and differentiation to large mature megakaryocytes. Excessive platelet numbers are released into the blood subsequently [1]. Although updated diagnostic criteria have been presented by Murphy et al. [2], ET still remains a diagnosis of exclusion.

Recently, several groups [3–6] have cloned thrombopoietin (TPO), the ligand of the c-Mpl receptor, and identified its role in regulating megakaryocytopoiesis and platelet production. To estimate serum TPO concentrations, three types of enzyme-linked immunosorbent assay (ELISA) have been established [7–9]. A sandwich ELISA described by Tahara et al. [9] appears to be the most sensitive of the three methods. This ELISA uses a mouse monoclonal antibody (Ab) as the capture Ab and a biotinylated rabbit polyclonal Ab as the detector; this ELISA is highly reproducible and specific. There was no cross-reaction with other blood components or cytokines to produce false-positive results. The detection limit of this ELISA is lower than the normal range.

Several groups have reported elevated serum TPO levels in ET but found no association between serum TPO levels and platelet counts [10–12]. In this study we measured a large number of serum TPO levels in ET using this highly sensitive sandwich ELISA and have now elucidated a relationship between serum TPO levels and platelet counts.

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TABLE I. Previous Treatment in 50 ET Patients at the Time of Their First TPO Measurement

Previous treatment	No. of patients
Untreated	23
Busulfan	17
Hydroxycarbamide	6
Carboquone	2
Ranimustine	1
Interferon	1
Total	50

MATERIALS AND METHODS

Patients and Controls

Serum samples were obtained from 50 patients with ET (17 males and 33 females) and 29 healthy volunteers (18 males and 11 females) after obtaining informed consent from each. In ET, the second TPO measurements were performed in 9 patients with different platelet counts. Patients with ET were diagnosed according to the criteria of the Polycythemia Vera Study Group [13]. The median age was 60 years [range 25 to 92] in ET patients. Table I shows the previous treatment of these 50 ET patients at their first TPO measurement. Twenty-seven of these patients had already been treated with chemotherapy (17 with busulfan, 6 with hydroxycarbamide, 2 with carboquone, 1 with ranimustine, and 1 with interferon), and the remaining 23 patients had been untreated at their first TPO measurement.

Measurement of Serum TPO by ELISA

Serum was separated by immediate centrifugation, and all samples were frozen at −80°C until use. A monoclonal Ab, termed TN1, and a polyclonal Ab to recombinant human TPO (rh TPO) were generated as previously described [9]. Serum TPO levels were determined by a sandwich ELISA using these antibodies [9]. Briefly, each of the 96 wells of flat-bottomed microtiter plates (Maxisorp, Nunc) was coated at 4°C overnight with TN1 at a concentration of 10 μg/mL in carbonate buffer (pH 9.4). After preincubation of the wells with a blocking reagent (Superblock in TBS, Pierce) for 30 min at room temperature, 100 μL of serum was added to each well and reacted with the coated TN1 overnight at room temperature. After washing with 20 mM Tris-HCI containing 0.5 M NaCl, 0.05% Tween-20, and 0.1% NaN_3, pH 7.5 (T-TBS), 100 μL of the biotinylated anti-rhTPO F $(ab')_2$Ab at 500 ng/mL in T-TBS containing 1% BSA and 2% polyethylene glycol (PEG 6000; dilution buffer) was added to each well for 3 hr at room temperature. After the solution was washed with T-TBS, 100 μL of streptavidin-alkaline phosphatase conjugate (Boehringer Mannheim) was added for 1 hr at room temperature. The color was developed using a kit for alkaline phosphatase (Gibco BRL). H_2SO_4 (0.3 M) was added to stop the reaction. Color intensity was measured by a plate reader at 492 nm. The absorbance of each sample was subtracted from that of the sample incubated with TN1. The sample concentration was calculated by regression analysis from a standard curve.

Statistical Analysis

The values for each measured variable are given as mean values ± standard deviation (SD). Spearman's rank correlation test was used in the calculation of correlations between serum TPO levels and platelet counts. Mann-Whitney's non-parametric test was used for the calculation of differences between groups.

RESULTS

Serum TPO Levels and Platelet Counts in Healthy Volunteers

Serum TPO levels from 29 normal individuals (18 males and 11 females) were measured. The values ranged from 0.25 to 1.72 (mean ± SD, 0.83 ± 0.36) fmol/mL. No statistical difference in serum TPO levels between males and females was noted ($P = 0.753$). There was no statistically significant correlation between serum TPO levels and platelet counts in normal individuals ($R = 0.074$, $P = 0.694$).

Serum TPO Levels in Patients With ET

Serum TPO levels and platelet counts ranged from 0.23 to 12.37 (2.70 ± 2.74) fmol/mL and from 162 to 1548 (670 ± 373) × 10^9/L, respectively. As shown in Fig. 1a, the mean serum TPO level in the 59 samples from patients with ET was significantly higher than in the normal ($P < 0.0001$). In the 23 ET patients who had been untreated, serum TPO levels and platelet counts ranged from 0.38 to 3.80 (1.33 ± 0.75) fmol/mL and from 641 to 1416 (946 ± 244) × 10^9/L, respectively. Figure 1b shows that the mean serum TPO level in these 23 patients also was significantly higher than in normal controls ($P = 0.007$). After chemotherapy, platelet counts became normal (<350 × 10^9/L) in 17 ET patients. Their serum TPO levels ranged from 1.48 to 12.37 (5.46 ± 3.68) fmol/mL. Thus, the mean serum TPO level in these 17 patients was higher than in the control group as shown in Fig. 1c ($P < 0.0001$).

Relationship Between Serum TPO Levels and Platelet Counts in ET Patients

Figure 2 shows serum TPO levels and platelet counts in ET patients and normal individuals. In ET patients, a strong inverse correlation was found between serum TPO levels and platelet counts ($R = -0.729$, $P < 0.0001$). In 23 untreated patients there was no significant correlation between serum TPO and platelet counts.

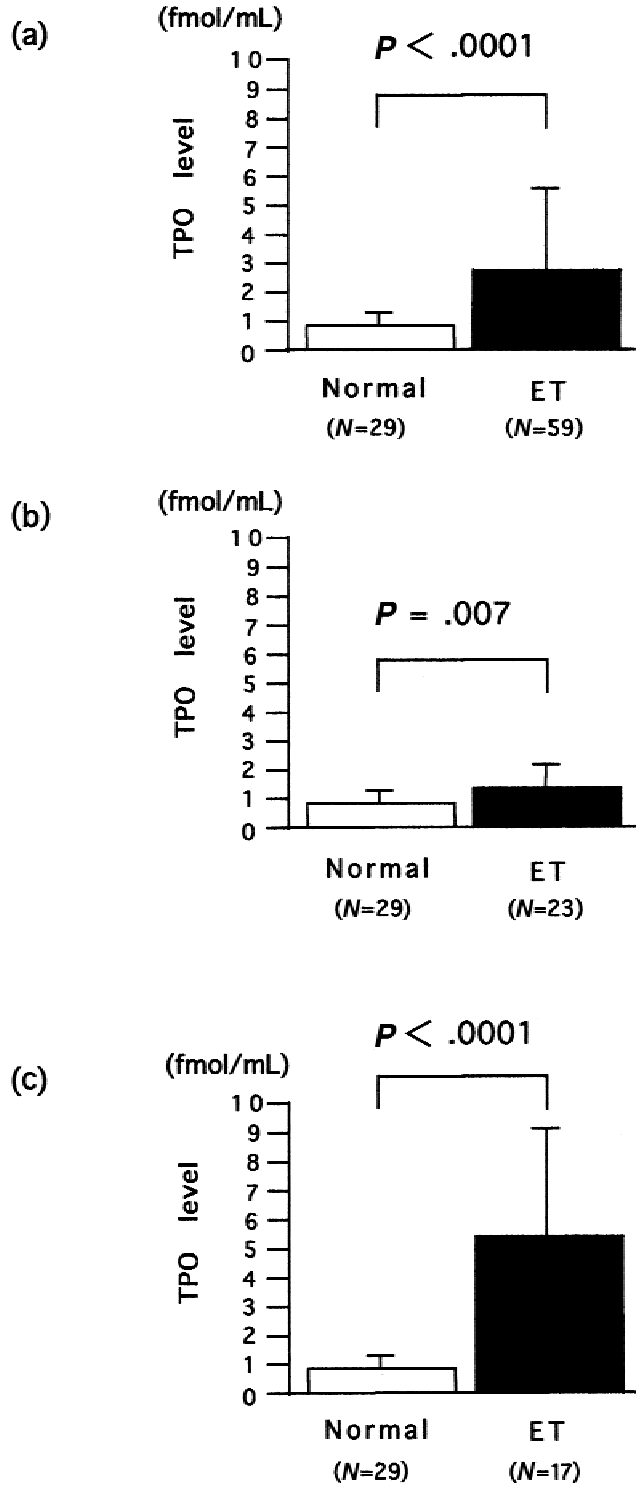

Fig. 1. (a) Serum TPO level in ET patients vs. normal individuals. The mean serum TPO level of 59 measurements from 50 ET patients was significantly higher than that from normal individuals ($P < 0.0001$). (b) Serum TPO level in 23 previously untreated ET patients vs. normal individuals. The ET mean TPO level was significantly higher than in normal individuals ($P = 0.007$). (c) Serum TPO level in 17 ET patients with normal platelet counts vs. normal individuals. The ET mean TPO level was significantly higher than in normal individuals ($P < 0.0001$).

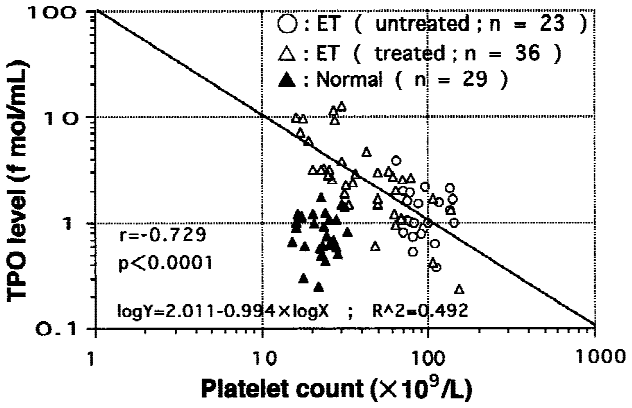

Fig. 2. Relationship between serum TPO level and platelet count. Individual values from untreated ET (○), treated ET (△), and normal individuals (▲) are shown. No statistically significant correlation was found between serum TPO levels and platelet counts in normal individuals. The straight line indicates the regression equation for ET only ($N = 59$; untreated and treated). A strong inverse correlation was seen between platelet count and serum TPO level ($R = -0.729$, $P < 0.0001$) in the ET group.

Two-Point Measurements With Different Platelet Counts in ET Patients

In nine cases, TPO levels were measured at two points with different platelet counts. Figure 3 shows the relationship between serum TPO levels and platelet counts. With platelet reduction, serum TPO levels became higher in all cases. Of the nine cases, four patients were treated with busulfan at the second measurement and untreated at the first measurement. In four other patients treatment had already been started with busulfan at the first measurement. The remaining patient had been treated with interferon at the first measurement and had a higher platelet count at the second measurement, indicating recurrence after interferon therapy.

DISCUSSION

It has been reported that human hepatocytes and proximal convoluted tubule cells in the kidney are the major sites of TPO production [14]. In the thrombocytopenic human subjects, TPO mRNA expression in the liver and kidney is not increased. By contrast, TPO mRNA expression in bone marrow stromal cells is upregulated in thrombocytopenic humans although this was not seen in the steady state using in situ hybridization [14]. Similar results have been reported in mice using semiquantitative reverse transcriptase-polymerase chain reaction [15,16]. These findings suggest that serum TPO levels are regulated by a feedback system at the level of gene expression, at least in human bone marrow stromal cells. However, it has also been suggested that serum TPO levels are regulated by platelet mass in animals [17,18]. At the

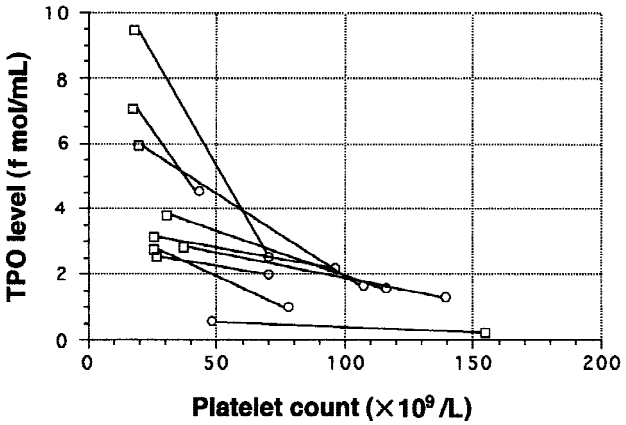

Fig. 3. Two-point measurements of serum TPO levels with different platelet counts in individual ET patients. Values from the first (○) and second (□) measurements are shown. A straight line between points indicates the same individual.

same time, serum TPO levels are extremely high in patients with aplastic anemia or chemotherapy-induced bone marrow hypoplasia but normal or only slightly elevated in patients with idiopathic thrombocytopenic purpura with megakaryocyte hyperplasia [19]. These clinical findings suggest that serum TPO levels are regulated not only by platelet mass but also by megakaryocyte mass, i.e., the total mass of c-Mpl in humans. These two mechanisms may regulate serum TPO levels in the steady or thrombocytopenic state.

In ET, several studies have reported elevated serum TPO levels compared with those in normal subjects [10–12]. However, those observers did not find correlation between serum TPO level and circulating platelet count. Similar data has been reported in myeloproliferative disorders including ET [20]. Our study found serum TPO levels in ET patients to be significantly higher than in normal individuals; moreover, there was a strong inverse correlation between serum TPO level and platelet count in ET patients. The difference between our study and others may be attributable not only to the large number of ET patients we examined but also to the method we used for measuring serum TPO.

The ELISA employed in this study is highly sensitive; TPO levels from 0.1 to >20.00 fmol/mL can be measured [9]. It is reproducible and specific. There was no cross-reaction with other blood components or cytokines. Even normal subjects have significant amounts of TPO in serum, ranging from 0.25 to 1.72 fmol/mL. In this study, the mean serum TPO level in the 23 ET patients who had been untreated was significantly higher than in normal individuals. If we assume that anti-platelet drugs, such as aspirin or ticlopidine, have no influence on the number of c-Mpl on platelets and megakaryocytes, the serum TPO level in ET is essentially higher than normal. Furthermore, when chemotherapy reduced the circulating platelet count, the serum TPO level became more elevated in the treated patients; in one case, TPO levels declined with recurrence of ET and thrombocytosis after interferon therapy. These cases also strongly support the inverse correlation between serum TPO level and platelet count in ET. In addition, 17 ET patients with normal platelet counts after chemotherapy had extremely high serum TPO levels. This finding further suggests the potential for recurrence.

It is of interest that serum TPO levels in ET are not down-regulated, despite the fact that, in myeloproliferative disorders such as polycythemia vera and chronic myeloid leukemia, serum erythropoietin and serum G-CSF are down-regulated with elevations in red cell and granulocyte counts, respectively. In ET, a clonal disorder with the potential for leukemic transformation, the role of the TPO–c-Mpl system has not been fully characterized. Several qualitative data have been reported recently. Kiladjian et al. found that relative expression of c-Mpl isoforms in ET and normal individuals was identical, and no mutation was detected in the clonal platelet populations derived from four ET patients [21]. Horikawa et al. [22] showed that expression of platelet c-Mpl protein and mRNA was dramatically reduced and c-Mpl-mediated signaling was not activated in platelets from ET patients. Quantitative expression of the megakaryocyte c-Mpl receptor in ET is unknown. In the clinical course of ET, the number of bone marrow megakaryocytes parallels the platelet counts before and after therapy. Most recently, in the familial ET generally inherited in an autosomal-dominant fashion, elevated TPO was identified as the primary event evoking thrombocytosis [23,24]. The level of those familial cases seems much higher than our sporadic cases. Furthermore, in view of its clonal origin, overproduction of TPO is unlikely to be involved in the pathogenesis of sporadic ET. Therefore it is reasonable to assume that total platelet and megakaryocyte mass, i.e., the total number of c-Mpl, plays a role to regulate serum TPO levels.

We found a strong inverse correlation between platelet count and serum TPO level in ET patients using a highly sensitive sandwich ELISA.

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